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Beyond water homeostasis: Diverse functional roles of mammalian aquaporins

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Keywords: aquaporin, solute transport, ion transport, membrane trafficking, cell volume regulation

The abbreviations used are: GLP, glyceroporin; MD, molecular dynamics; SC, stratum corneum; ANP, atrial natriuretic peptide; NSCC, non-selective cation channel; RVD/RVI, regulatory volume decrease/increase; TM, transmembrane; ROS, reactive oxygen species

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Abstract

BACKGROUND: Aquaporin (AQP) water channels are best known as passive transporters of water that are vital for water homeostasis.

SCOPE OF REVIEW: AQP knockout studies in whole animals and cultured cells, along with naturally occurring human mutations suggest that the transport of neutral solutes through AQPs has important physiological roles. Emerging biophysical evidence suggests that AQPs may also facilitate gas (CO_2) and cation transport. AQPs may be involved in cell signalling for volume regulation and controlling the subcellular localization of other proteins by forming macromolecular complexes. This review examines the evidence for these diverse functions of AQPs as well their physiological relevance.

MAJOR CONCLUSIONS: As well as being crucial for water homeostasis, AQPs are involved in physiologically important transport of molecules other than water, regulation of surface expression of other membrane proteins, cell adhesion, and signalling in cell volume regulation.

GENERAL SIGNIFICANCE: Elucidating the full range of functional roles of AQPs beyond the passive conduction of water will improve our understanding of mammalian physiology in health and disease. The functional variety of AQPs makes them an exciting drug target and could provide routes to a range of novel therapies.

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1. Introduction

The aquaporin (AQP) protein family is comprised of many small integral membrane proteins found in all phylogenetic kingdoms. There are up to 13 mammalian AQPs, which are found in most tissues with functions ranging from the regulation of renal water balance [1], brain-fluid homeostasis [2], triglyceride cycling between adipocytes and the liver [3] and structural integrity of the eye lens [4]. Because of this, understanding AQP function is crucial for the study of healthy ageing as well as the onset of many disease states such as brain swelling following stroke or head injury [5], nephrogenic diabetes insipidus [6, 7], cataracts [8], obesity [9], cancer cell proliferation and migration [10] and tumour angiogenesis [11]. Many of these functions and diseases involve either permeability of molecules other than water, or a function of the AQP other than facilitating membrane permeability.

Thirteen human AQPs have been discovered to date; they range in size (before post-translational modification) from 27 kDa (AQP8) to 37 kDa (AQP7) and, for those proteins for which single channel permeability has been quantified, have a 100-fold range in water permeability. A sub-set of these AQPs also function as channels for glycerol (and other solutes) and are referred to as (aqua)glyceroporins (GLPs). In humans, these are AQP3, -7, -9 and -10. AQP6 has also (controversially, see section 4) been shown to be permeable to glycerol, although phylogenetically it is a member of the water-selective AQP subfamily [12]. It also is notable for its unusual permeability properties (activation by low pH and anion permeability) and intracellular localization. The permeability properties of the mammalian AQPs are summarized in Table 1.

This review discusses how AQPs select and regulate the passage of solutes such as glycerol, urea and ammonia across cell membranes and the physiological relevance of this solute flow as well as putative functions of AQPs beyond facilitation of membrane permeability.

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AQP	Single channel water permeability ($\times 10^{-14}$ $cm^3 s^{-1}$)	Glycerol permeability	Urea permeability	Ammonia permeability
AQP0	0.25 [13]; gated by calmodulin [14]; X, PL	No [13]; X	No [15]; X	No [16]; X
AQP1	6.0 [13]; X	No [13]; X	No [17]; X	No [17]; X
AQP2	3.3 [13]; X	No [13]; X	No [15]; X	No [16]; X
AQP3	2.1 [13]; X	Yes [13]; X	Controversial [15, 17-19]; X	Yes [16, 17]; X
AQP4	24 [13]; X	No [13]; X	No [20]; X	No [16]; X
AQP5	5.0 [13]; X	No [13]; X	No [21]; X	No [16]; X
AQP6	Low pH and Hg^{2+} induce water permeability [22, 23]. Basal permeability either is very low [23, 24] or zero [25, 26]; X	Yes** [26]; X	Yes** [26]; X	Yes [16]; X
AQP7	Permeable [27]; no quantitative data; X	Yes [27]; X	Yes [27]; X	Yes [16]; X
AQP8	Permeable [28, 29]; no quantitative data; X, PL	No [28, 29]; X, PL	Controversial [28-30]; X, PL	Yes [17, 31]; X, Y
AQP9	Permeable [32]; no quantitative data; PL	Yes [17, 32]; X, PL	Yes [17, 32]; X, PL	Yes [17]; X
AQP10	Permeable [33]; no quantitative data; X	Yes [33]; X	Yes [33]; X	Unknown
AQP11	Permeable [34],~2 [35]*; M, V	Yes** [36]; M	Unknown	Unknown
AQP12	Unknown	Unknown	Unknown	Unknown

Table 1 – Water, glycerol, urea and ammonia (NH_3) permeabilities of known human AQPs. Quantitative water permeability is per AQP channel i.e. after subtraction of the basal permeability of the expression membrane and normalized to the number of AQP molecules. Expression systems: X – Xenopus *oocytes, PL – proteoliposomes, M – mammalian cells, V – vesicles derived from membrane fragmentation of AQP-expressing mammalian cells, Y – ammonia transport deficient yeast. . *estimated from membrane fragment vesicle water permeability relative to AQP1-containing vesicles and relative expression level. **These data have not been independently replicated.*

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2. The structural biology of the AQP family is established

There is a large amount of medium-to-high-resolution structural data available for the AQP family (43 structures deposited in the Protein Data Bank for 11 different AQPs). These structures suggest that the AQPs share many common structural features. The AQP monomer is composed of six tilted transmembrane (TM) helices that surround a central cavity containing two helix-forming loops that enter and exit from the same side of the membrane (see Figure 1A). This central cavity is the pathway for water and solute transport. The two helical loops contain highly conserved asparagine-proline-alanine (NPA) motifs. Crystallography and molecular dynamics (MD) simulations suggest that the asparagine residues act, in concert with surrounding backbone carbonyl groups, to enable water transport by providing hydrogen bonds to water molecules [37], thereby lowering the free energy penalty (caused by breaking of water-water hydrogen bonds) of removing a water molecule from bulk solution. Recent biophysical experiments comparing AQPs with gramicidin and the potassium channel KcsA [38] suggest that the number of pore-lining hydrogen-bonding sites for water determines the channel water permeability. In addition to providing hydrogen-bonding sites, the asparagine residues help to orient the water molecules such that the hydrogen atoms point 'outwards' from the pore, creating a barrier to proton transport [39]. A recent sub-angstrom crystal structure of the *Pichia pastoris* AQY1 found overlapping water densities in the pore that, supported by MD simulations, suggested a correlated pairwise movement of water molecules through the extracellular end of the pore. This correlated motion may help to minimise water-water hydrogen bonds in this region, providing a further barrier to proton permeation, without compromising water transport [37].

Crystallographic analysis of the *E. coli*, GlpF (a GLP), suggests that the same asparagine residues also form hydrogen bonds with glycerol [40]. This is likely to be also true of other polar solutes that permeate GLPs.

AQPs have intracellular amino (N-) and carboxyl (C-) termini of varying lengths; AQP8 has a short C-terminus of 10-15 residues, whereas AQP4 has a large C-terminus consisting of ~70 residues. AQP11 has a large N-terminal tail of ~40 residues, whereas AQPs 1,2 and 5 have short N-termini of <10 residues. In contrast to the structural conservation of the TM region of AQPs, much less is known about the structure of the termini. This is because the majority of AQP crystal structures are obtained using constructs in which the N- and C-terminal tails of the protein have been truncated due the difficulty of obtaining well-diffracting crystals using full-length AQP molecules [41]. This suggests an inherent structural flexibility in these regions of AQP molecules and indeed a recent X-ray structure of AQP2, which included ~20 residues of the (truncated) C-terminal tail, found this fragment of the C-terminus in four strikingly different conformations in each of the four AQP2 monomers within the tetrameric unit cell [42].

AQPs are generally agreed to form tetramers [43-45] assembled around a central axis perpendicular to the plane of the membrane (see Figure 1B). A fifth, hydrophobic pore appears to form around this central axis with each of the monomers contributing one of the four 'walls' of the pore. The function, if any, of this fifth pore is not yet known, although postulated functions will be discussed in later sections. Some biochemical studies have suggested that tetramers formed by GLPs have reduced stability compared to tetramers formed by AQPs with strict water selectivity [46, 47].

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3. The principles of solute selectivity by AQPs remain to be established

All AQPs (except AQP12, which has yet to be characterized) transport water (Table 1), while some also transport glycerol. The glycerol permeable GLPs (e.g. human AQPs 7, 9 and 10) are also permeable to urea. The urea permeability of AQP3 is controversial, with some studies reporting urea transport and others no transport [15, 17-19]. This may be due to methodological differences, and is discussed in detail in section 4.2.1.

Of the mammalian GLPs AQP3, -7, and -9 are also permeable to ammonia. AQP8 is the only glycerol impermeable mammalian AQP that is permeable to ammonia (excluding 11 and 12, whose glycerol and/or ammonia permeabilities are currently unknown). The ammonia permeability of the most recently discovered member of the GLP group, AQP10, is still unknown.

AQP11 and -12 are the most recently discovered members of the AQP family [48, 49]; due to this and their localisation to intracellular membranes, non-water permeability experiments and functional studies have yet to be reported for AQP11 and AQP12 respectively. There is one report that AQP11 increased the glycerol permeability of an adipocyte cell line [36], but this permeability of AQP11 has yet to be replicated.

The 'size exclusion' model of AQP selectivity posits a correlation between the size of channel entrance and the permeability of the channel to different polar molecules. Notably, this model is based on *in silico* and crystallographic experiments, but minimal *in vitro* experiments; it is therefore unclear whether this model is broadly applicable to the AQP family as a whole. A conserved arginine residue (in the second helical loop directly after the NPA motif) forms part of a channel constriction known as the aromatic/arginine (ar/R) selectivity filter (see Figure 1A). In water-selective AQPs, the other components of this filter are a phenylalanine in the top half of the pore-facing side of transmembrane helix 2 (TM2) and a histidine in a similar position in TM5. In glycerol and urea permeable AQPs, this histidine is replaced by a small amino acid residue such as glycine (AQP3, -7, -10) or alanine (AQP9), although if the histidine is mutated to alanine in AQP1 it does not become a glycerol channel. If both aromatic members of the filter (H and F) are mutated to alanine, AQP1 functions as a urea channel and to a lesser extent as a glycerol channel [50]. Based on this observation and molecular simulations of AQP1 and the *E. coli* GLP, GlpF [50, 51], it has been suggested that the cross-sectional area of the pore at the ar/R region determines AQP selectivity for neutral polar solutes. Additionally, based on crystallographic analysis of GlpF and the bacterial water-selective AQP, AQPZ, it has been suggested that the positioning of the ar/R residues by the surrounding unstructured loops has a role in determining the channel size [52]. In AQP8, the only glycerol-impermeable, ammonia-permeable mammalian AQP, the third member of the filter is likely an isoleucine residue (based on sequence alignment), which is slightly larger than alanine or glycine. Overall, current thinking on solute selectivity suggests that the aromatic residues of the ar/R filter are important for solute exclusion in the water selective AQPs, but further molecular factors may be involved in mediating solute selectivity.

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4. Physiological solute transport by AQPs

4.1 Glycerol transport

The movement of glycerol around the body is predominantly thought to include glycerol release from fat tissue through AQP7, entry into the liver through AQP9 and movement in the skin and kidneys through AQP3.

4.1.1 AQP7

AQP7 is expressed in adipose tissue in adipocytes [53] and capillary endothelia [54]. Upon hypoglycaemia, triglycerides are broken down within adipocytes to glycerol and free fatty acids [55]. Adrenaline, the plasma level of which is elevated upon hypoglycaemia, caused translocation of AQP7 from intracellular membranes to the plasma membrane [53]. Adrenaline signals through the β3-adrenergic receptor to initiate adipocytic lipolysis [56] making this the most likely link between adrenaline and AQP7 translocation although this has yet to be verified. Recent work has also suggested that AQP7 can be relocalized to lipid droplets by noradrenaline, possibly in a PKA dependent manner [57], raising the possibility of differential effects of lipolysis-inducing hormones on AQP7 localization.

The glycerol liberated from triglyceride storage is released from adipocytes [58] and plasma glycerol becomes elevated. In AQP7 -/- mice, adipocyte plasma membrane glycerol permeability was reduced threefold [59]. Plasma glycerol levels in the fasting state and in response to β3-adrenergic agonist were reduced and adrenaline-induced glycerol secretion by cultured mouse adipocytes (differentiated 3T3-L1 cells) was reduced approximately two-fold [60]. These results suggest that the major pathway for glycerol efflux from adipocytes after lipolysis is AQP7.

AQP7 -/- mice develop adipocyte hypertrophy and subsequent obesity in adulthood[59]. A loss of function mutation in the human AQP7 gene has been discovered (G264→V) although this genotype was not correlated with obesity in humans [61]. This may be due to the presence of AQP10 in human adipocyte membranes, which was found to contribute ~50% of the water and glycerol permeability of healthy human adipocyte plasma membrane vesicles [62]. Mouse AQP10 is a pseudogene [63] and therefore this secondary route for glycerol efflux does not exist in mice.

The serum concentration of glycerol in humans under normal physiological conditions is typically between 0.05 and 0.1 mM [64] and increases several-fold upon fasting (due to glycerol and free fatty acid liberation from adipose triglyceride storage) [65]. Glycerol is almost completely reabsorbed by the kidneys [66] (unless it is raised above ~0.3 mM[67]), suggesting the existence of a glycerol reabsorption pathway. AQP7 is expressed in the proximal tubule of the kidney. AQP7 -/- mice showed marked glyceroluria in comparison to wild type (~400-fold increase in urine glycerol) [68]. Human children homozygous for the AQP7 G264V mutant were found to have hyperglyceroluria, with a ~1000-fold increase in urine glycerol when compared to heterozygous familial controls [69]. The G264V mutant has been shown to have no activity as a glycerol or water channel in Xenopus oocytes [61], although plasma membrane expression was not verified in this study so that it could not differentiate between a non-functional channel and an incorrectly localised channel. The mutation disrupts a conserved GxxxG motif. These are important motifs for

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transmembrane helix-helix interactions because they allow close contact between the backbone atoms of the two helices of an interacting pair [70]. It may be that the protein is incorrectly trafficked and localised in intracellular membranes due to an inability to form tetramers or an interaction required for membrane trafficking. Regardless of the loss of function mechanism, these data strongly suggest that AQP7 is the TM glycerol reabsorption pathway in the renal proximal tubule.

4.1.2 AQP9

AQP9 is expressed in the liver [71], primarily in the hepatocytes with the strongest expression at the sinusoidal surface [72] (the liver sinusoids are continuous with the hepatic artery and portal vein). Upon fasting, glycerol released into the plasma from adipocytes is taken up by hepatocytes and used as a substrate for gluconeogenesis [73].

In rats, AQP9 protein expression is increased up to 20-fold after 24-96 hours fasting [32]. In mice, AQP9 expression measured in purified hepatocyte plasma membrane vesicles increased 10-fold after 18 hours fasting. This 10-fold increase was accompanied by a two-fold increase in hepatocyte plasma membrane glycerol permeability, which was reversed by addition of phloretin (an apple polyphenol that inhibits AQP9, AQP3 and several non-AQP membrane channel proteins including the SGLT1&2 glucose transporters and UT-A urea channels [74]). The increase was abolished in AQP9 -/- mice [75]. Fasted AQP9 -/- mice have elevated plasma glycerol compared to wild-type [75, 76]. In addition, plasma glucose concentration was decreased [76], indicating a gluconeogenetic deficiency. This data supports a physiological role for AQP9-mediated glycerol uptake by hepatocytes during fasting-induced hypoglycaemia.

Although the AQP9-mediated diffusion of glycerol into hepatocytes forms a sizeable proportion of the glycerol uptake in the fasting state (~50% in fasting mice), the fact that the membrane permeability to glycerol only doubles upon such a large increase in AQP9 expression suggests that there is also an AQP9-independent route for glycerol uptake. Whether this is directly across the lipid bilayer or through a phloretin insensitive glycerol channel is not clear.

$CD8^+$ (cytotoxic) T cells are white blood cells that facilitate the destruction of infected or otherwise damaged cells. During infection, antigen-specific $CD8^+$ cells proliferate and differentiate into effector cells, which are involved in combatting infection. After pathogen clearance, most of the effector cells die and a small population remains as memory T cells, which can survive for decades [77]. In mouse memory cells, AQP9 expression was upregulated post-infection via IL-7 signalling, allowing cells to import glycerol for triglyceride synthesis. AQP9 -/- T cells had reduced long-term survival compared to +/+ cells and the long-term survival of the +/+ cells could be inhibited by phloretin [78]. This suggests that AQP9 expression can act as a metabolic switch, enabling long-term survival of memory T cells by enabling triglyceride synthesis to build up an energetic reserve, allowing cell survival in nutrient poor conditions.

Similarly to that seen in hepatocytes, intracellular glycerol in $CD8^+$ T cells was reduced by ~50% in the AQP9 -/- cells, again suggesting a secondary route for glycerol uptake.

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4.1.3 AQP3

AQP3 is expressed in the skin in keratinocytes below the stratum corneum (SC). It is most strongly expressed at the plasma membrane in these cells with some intracellular labelling reported in cells of the basal layer [79]. The ability of the epidermis to maintain hydration is impaired in AQP3 -/- mice. In dry conditions, AQP3 -/- mice showed comparable (reduced) levels of SC hydration to wild-type mice, whereas at normal humidity, SC hydration was lower in the AQP3 -/- mice [80]. Elevated humidity (which prevents water loss via evaporation) did not correct the deficiency, which suggests that the primary role of AQP3 in skin hydration is not to provide water to replace that lost by evaporation.

SC glycerol concentration in AQP3 -/- mice is reduced to ~40% of that of wild-type, with no significant difference in the levels of other osmolytes (ions, glucose, urea, lactate and free amino acids) [81]. Glycerol acts as a humectant (a 'water-retaining' osmolyte), which may be the mechanism by which it maintains skin hydration. It has also been suggested that glycerol may prevent SC water loss by inhibiting the phase transition of intercellular lipids from the liquid crystalline to the solid phase [82] due to the fact that breaks in a solid crystal lattice increase the water permeability in *in vitro* models of the SC intercellular lipid barrier [83]. The rate of transport of glycerol from blood to the SC was reduced in AQP3 -/- mice resulting in reduced lipid biosynthesis[84]. This suggests a further role for glycerol in maintaining skin hydration by allowing the maintenance of the SC lipid barrier.

Further evidence for the role of AQP3-mediated glycerol transport is provided by the fact that topical or systemic administration of glycerol was found to correct the skin deficiencies in AQP3 -/- mice [84].

4.2 Urea transport

Urea is produced in the liver (as a non-toxic carrier of waste nitrogen) from ammonia, which is a neurotoxic product of protein degradation. Ammonia causes cell death of astrocytes by stimulating the mitochondrial permeability transition [85] (opening of promiscuous mitochondrial membrane channels that leads to apoptosis or necrosis, depending on cellular ATP availability [86]), although the mechanism by which this happens is not clear. An adult human excretes about 25 g/day of urea in the urine, and urea transport in the kidney is vital for the urinary concentrating mechanism [87]. The physiological roles of urea transport by AQPs are less clear than those of water and glycerol transport.

4.2.1 Which mammalian AQPs are urea channels?

AQPs 7 [27], 9 [88] and 10 [33] have been shown to be urea permeable and there is a consensus in the literature on the urea permeability of these AQPs. For example, AQP9 is a urea channel in the liver expressed in hepatocytes at the sinusoidal surface. Urea permeability measurements performed on mouse hepatocyte plasma membrane vesicles of AQP9 -/- and urea transporter type A1/3 (UT-A1/3) -/- mice showed that AQP9 contributes ~30% to mouse hepatocyte membrane permeability and a member(s) of the UT-A family contributes ~40%[91]. However, AQP9 -/- mice did not demonstrate any deficiency in urea clearance from hepatocytes in a state that promotes elevated hepatic urea production (high protein diet), suggesting that AQP9 and UT-A provide redundant pathways for urea transport from hepatocytes to the blood.

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However, there is conflicting evidence in the literature on whether AQPs 3 and 8 are urea channels. Early work on AQP3 suggested that rat AQP3 was urea permeable, with expression of AQP3 in *Xenopus* oocytes increasing urea uptake twofold after 30 minute incubations of oocytes with radiolabelled urea [89] or threefold in oocyte swelling assays [18]. Further studies on rat AQP3 found no urea transport using similar oocyte volumetric techniques [15, 17]. These studies differed in that the former used 165 mM urea whereas the latter two used 20 mM urea. It may be that AQP3 urea transport is so slow that at 20 mM it does not induce large enough volume changes to be measured on the timescale of an oocyte swelling experiment (~1 min), or that the transport is non-linear, although this seems unlikely given the linear nature of water and glycerol transport by AQP3 [90].

In one study, human AQP3 was used a positive control for AQP urea permeability. 1 mM of urea was added to AQP3-expressing oocytes and after a 10 minute incubation, the oocytes had an intracellular urea concentration of ~75 μM (assuming an oocyte volume of 1 μL) [19]. This is ~10% of the expected equilibrium value, and the fact that it is still so far from equilibrium even after 10 minutes suggests that urea transport through AQP3, whilst non-zero, is very slow. This may explain the different results between short (typically ~1 min) volumetric experiments and the longer timescale radiolabelled solute uptake experiments.

Early work on mouse AQP8 suggested that it was urea permeable [30], whereas rat AQP8 was not [29], both using radiolabelled solute uptake measurements in *Xenopus* oocytes. Work on purified rat, mouse and human APQ8 in proteoliposomes suggested that neither rat nor human AQP8 were urea permeable [28]. This study did not report mouse urea permeability due to liposome swelling discrepancies, probably caused by the ionic detergent required to solubilize mouse AQP8.

There is considerable interspecies amino acid sequence variability for AQP8 (e.g. 74% identity between human and mouse AQP8, c.f. 94% for AQP1 and 93% for AQP4). Interestingly, one difference between human and mouse AQP8 is a residue that is predicted to be pore-lining (based on a homology model to bovine AQP0 [28]) and situated at the ar/R filter, G207 (in human; A205 in mouse). The idea of species-specific differences in permeability of AQP8 is intriguing, but studies performed in parallel in the same experimental system are required to validate this.

4.3 Ammonia transport

Ammonia is produced as a by-product of protein breakdown and quickly converted to urea via the hepatic urea cycle to prevent ammonia neurotoxicity. It is important for control of acid-base balance in the kidney, where ammonia synthesis and excretion are tightly regulated and change in response to acid- or alkalosis [92].
Members of the GLP subfamily, AQPs 3 [17], 7 [16] and 9 [17], have been reported to be permeable to ammonia, as has AQP8 [17, 93]. There is also evidence of ammonia permeability of AQPs 1, 6 and 7 using microelectrode measurements of oocyte surface pH[16]. The physiological relevance of ammonia permeability of AQPs is unclear.

AQP8 -/- mice have only the very mild phenotypic abnormality of slight hypertriglyceridaemia after three weeks on a high (50%) fat diet [94]. AQP8 is expressed in the inner mitochondrial membrane of hepatocytes [95] and increased the

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transport of an ammonia analogue into AQP8-expressing *S. cerevisiae* and rat hepatocyte mitochondria by three-fold. However, AQP8 -/- mice do not show any impairment in ammonia clearance under physiological conditions or when chronically or acutely loaded with ammonia [96]. This suggests a secondary (non-AQP) pathway for ammonia, which either provides the majority of ammonia permeability in these tissues, or is up-regulated in ammonia-permeable AQP knockouts. The proteins associated with the Rhesus (Rh) blood group system have been shown to function as ammonia channels [97]. Several of these are expressed in the liver [98] (RhB and RhC) and the kidneys (again RhB and RhC), where ammonia has an important role in acid-base balance [99]. Rh -/- mice have been generated, however these studies have focused on the erythrocytic Rh proteins [100, 101]. Phenotype analysis of RhB and RhC -/- organisms and double AQP/Rh knockouts could provide an answer. Knockdown of AQP8 in primary rat hepatocytes by ~80% reduced ammonium chloride-induced ureagenesis by 30% and abolished glucagon-stimulated increases in ureagenesis [102]. AQP8 knockdown in a human proximal tubule cell line decreased the rate of ammonia excretion by 31% at pH 7.4 and by 90% at pH 6.9 [103], suggesting that AQP8 ammonia permeability might be required for renal ammonia excretion and be involved in the renal adaptive response to acidosis. Furthermore, acid-induced downregulation of AQP8 by 30% in primary rat hepatocytes was correlated with a 31% reduction in hepatocyte ureagenesis, and AQP8 downregulation was correlated with reduced liver urea content in rats subjected to seven days of acidosis [104]. These data support the idea of a physiological role for AQP8 in either plasma membrane diffusion of ammonia, mitochondrial ammonia transport, or both in support of renal and hepatic ammonia handling.

AQP3 -/- mice have several physiological abnormalities including reduced skin elasticity [80] and polyuria [105], but these can all be explained in terms of reduced glycerol and water permeability, so it is unclear whether AQP3 ammonia permeability has any physiological function.

AQP7 is present in adipocytes. It has been shown in humans that during intense exercise, adipose tissue removes ammonia from the plasma and increases its glutamine/glutamate ratio[106], suggesting incorporation of ammonia into glutamine through glutamine synthase as a secondary detoxification pathway in support of the hepatic urea cycle at times of elevated plasma ammonia. Whether AQP7 contributes to this ammonia uptake is yet to be investigated.

4.4 Carbon dioxide transport

The majority of carbon dioxide (CO_2) produced by cellular metabolism (~70%) is transported to the lungs for expulsion from the body via the bicarbonate (HCO_3^-) system. Briefly, carbon dioxide diffuses out of the cells in which it is produced and into the plasma. It moves down its concentration gradient into erythrocytes (red blood cells), where carbonic anhydrase catalyses conversion into carbonic acid. Upon dissociation ($H_2CO_3 \rightarrow H^+ + HCO_3^-$), the HCO_3^- ion is exchanged across the erythrocyte membrane for a chloride ion and the proton binds to haemoglobin [87].

There has been speculation that CO_2 transport across the plasma membrane of erythrocytes might be aided by channel proteins [107, 108]. There is conflicting data on whether an AQP contributes to this potential CO_2 pathway. AQP1 is expressed at the plasma membrane of erythrocytes [109]. Human AQP1 has been reported to

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increase the CO_2 permeability of Xenopus oocytes four-fold in the presence of carbonic anhydrase suggesting that AQP1 functions as a CO_2 channel [110]. Further work ruled out the possibility of AQP1 increasing the oocyte membrane permeability by altering local lipid composition or structure, interaction between AQP1 and carbonic anhydrase or up-regulating a native CO_2 channel by use of a mercurial AQP1 inhibitor (pCMBS) and the AQP1 C189S mutant, which is insensitive to mercury [111].

AQP1 -/- human erythrocytes (Colton-null cells) retain HCO_3^- permeability, but have a 60% reduction in CO_2 permeability when compared to human erythrocytes expressing AQP1 (i.e. not Colton-null) [112]. However, the CO_2 permeability of AQP1 -/- mouse erythrocytes from AQP1 knockout mice was not different to wild-type [113].

Stopped flow experiments with AQP1 reconstituted into liposomes have also given conflicting results. AQP1 from human blood increased the CO_2 permeability of liposomes four-fold[114], whereas mouse AQP1 showed no increase[113]. This agrees qualitatively with the results from measurements on intact erythrocytes in that human AQP1 appears to increase CO_2 permeability of the membrane, whereas mouse AQP1 does not. This may be due to methodological differences, but it also raises the intriguing possibility that human AQP1 is CO_2 permeable whereas mouse AQP1 is not.

It is possible that if CO_2 permeates AQP1, it does so through the central tetrameric pore rather than the monomeric water pore. Molecular dynamics simulations using a variety of enhanced sampling methods and unbiased simulation consistently suggest that the free energy barrier to permeation of the central, hydrophobic pore of AQP1 is considerably smaller than the barrier to permeation of the water pores [115-117], probably due to the water-protein hydrogen bonds at the selectivity filter and NPA sites that need to be disrupted in order for a CO_2 molecule to traverse the water pore. Simulation of model bilayers suggest that the free energy barrier for passage of CO_2 directly across the membrane is much smaller than for permeation through AQP1, and in this kind of system, AQP expression would probably serve to *decrease* the CO_2 permeability by reducing the surface area available for diffusion directly across the membrane [115, 116]. However, it is not clear how good of a model a single species phospholipid bilayer is for a cellular membrane. It is possible that a simulated bilayer containing a mixture of lipid species along with sterols would display different resistance to CO_2 passage, and biophysical measurements of CO_2 diffusion into artificial lipid vesicles showed that addition of cholesterol could reduce membrane CO_2 permeability by up to 100-fold [118]. This agrees qualitatively with MD simulations that include cholesterol [119]. In addition to CO_2, MD simulations have also suggested that the central pore could be permeable to molecular oxygen [115] and there is some evidence that AQP1 overexpression could accelerate cellular hypoxia [120], although whether this was a direct or indirect effect was not clear.

It seems apparent that AQP1 can conduct CO_2 (possibly along with AQP 0, 5, 6 and 9 and the M23 isoform of AQP4 [16]), however the physiological relevance (if any) of AQP CO_2 permeability is yet to be demonstrated. There is also a similar debate in the plant AQP field on whether AQPs might contribute to regulating CO2 levels for photosynthesis [121]. A recent review dedicated to AQPs and CO_2 permeability of

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biological membranes [122] concluded that "the debate about the mechanism of membrane CO2 diffusion continues and it is difficult to draw general conclusions". This is still very much an open question.

4.5 Hydrogen peroxide transport

Superoxide (O_2^-) molecules are produced in the mitochondria as a by-product of ATP synthesis (particularly by complexes I and III in the electron transport chain) and by the NADPH oxidase (NOX) family of enzyme complexes, which couple intracellular oxidation of NADPH to extracellular production of superoxide (O_2^-) molecules. High levels of reactive oxygen species (ROS) derived from these pathways are well known to cause cellular damage and even death and mitochondrial ROS were long considered to be purely harmful by-products of an imperfect metabolic system. However, work in the last decade has shown that they are crucial for a variety of physiological processes including adaptation to hypoxia, immune function and regulation of autophagy (probably mediated by redox modification of cysteine residues) [123], so clearly there is a need for tight regulation of ROS levels. Superoxide undergoes disproportionation to molecular oxygen and hydrogen peroxide (H_2O_2), which can be catalysed by the enzyme superoxide dismutase [124], and the highly reactive H_2O_2 can then go on to form a variety of further ROS.

H_2O_2 and H_2O have similar molecular sizes, dipole moments and hydrogen bonding capacities [125], therefore it would not be surprising to find that some AQPs could transport H_2O_2. The half-life for H_2O_2 in cells is very short (e.g. ~1 ms in lymphocytes [126]), making biophysical experiments of the kind used for e.g. glycerol or urea permeation very difficult. Growth assays using H_2O_2 transport-deficient yeast are typically used, and reduced growth after expression of an AQP is interpreted as an AQP-mediated increase in membrane H_2O_2 permeability causing cellular damage and/or metabolic disruption. Using this technique, human AQP8, rat (but not human) AQP1 and several mutants thereof were shown to be H_2O_2 permeable [127]. Based on this data, the authors suggested that all AQPs function as H_2O_2 channels. It is not clear whether this is a valid generalization, but clearly some mammalian AQPs are H_2O_2 channels.

Imaging studies using a novel H_2O_2 sensitive fluorescent dye in transiently transfected HEK293 cells demonstrated H_2O_2 permeability for human AQP8 and AQP3 (but not AQP1). Furthermore, AQP3 overexpression in HeLa cells allowed the cells to respond to serum starvation via ROS mediated activation of the AKT (protein kinase B) signalling pathway, whereas cells not expressing AQP3 could not. Knockdown of AQP3 in a colon cancer cell line inhibited the AKT response to epidermal growth factor (EGF) [128], which can initiate ROS signalling via activation of NOX complexes. This suggests that AQP3 is required for uptake of NOX-generated H_2O_2 in EGF signalling.

T cell migration along chemokine gradients is vital for correct immune responses. T cells from AQP3 -/- mice had 40-60% reductions in migration distance in response to a variety of chemokines and had a ~50% reduction in chemokine-induced transendothelial migration. Activation of Cdc42, a small GTPase involved in cytoskeletal reorganization associated with chemotaxis, was completely inhibited in the AQP3 -/- cells. Treatment with extracellular catalase, which catalyses H_2O_2 breakdown to H_2O and O_2, prevented the Cdc42 response in AQP3 +/+ cells and

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treatment with high concentration (100 μM) of exogenous H_2O_2 recovered both the Cdc42 response and the T cell migration in AQP3 -/- cells [129]. Together, these data suggest that AQP3 is required for uptake of extracellular H_2O_2 (probably NOX-generated) in the T cell chemotactic response.

Intracellular ROS levels can be elevated in leukaemia cells and NOX-derived ROS can activate leukaemia cell survival pathways. In a leukaemia cell line, shRNA-mediated knockdown of AQP8 reduced and overexpression increased intracellular H_2O_2 content as well as H_2O_2 uptake after 10 mins in 100 μM H_2O_2. Furthermore, cellular glucose uptake and proliferation were correlated with intracellular H_2O_2 and AQP8 expression [130], suggesting that AQP8 can facilitate increased metabolism and proliferation in leukaemia.

AQP8 is also expressed in inner mitochondrial membranes (IMM) in hepatocytes. Knockdown of AQP8 by 60% using siRNA in a hepatocyte cell line caused a twofold increase in mitochondrial ROS concentration and a 45% decrease in H_2O_2 output in isolated mitochondria. This was correlated with an 80% depolarization of the mitochondrial membrane, which was reversible by cyclosporin A (an inhibitor of the mitochondrial permeability transition) or a mitochondrial antioxidant, and a ~30% reduction in cell viability [131]. This suggests that AQP8-mediated H_2O_2 release from hepatic mitochondria acts as a mechanism to minimize mitochondrial oxidative stress.

AQP11 is localized to the ER in the renal proximal tubule. The C227S mutation of AQP11 caused proximal tubule injury and eventual renal failure in mice [132]. Mice heterozygous for the AQP11 C227S mutant were predisposed to glucose-induced accumulation of ROS in the proximal tubule and reduction of kidney function. Inhibition of glucose uptake by the non-specific inhibitor phlorizin or antioxidant (sulforaphane) treatment was able to protect kidney function. This effect was reproduced in a proximal tubule cell line, in which siRNA-mediated knockdown of AQP11 increased intracellular ROS concentration twofold in the absence of glucose and fourfold in the presence of glucose [133]. It is not clear whether AQP11 was controlling intracellular ROS accumulation by acting as an ER H_2O_2 channel, or whether this was an indirect effect.

Taken together, these examples provide clear evidence for AQP-mediated H_2O_2 membrane permeability and suggest physiological roles in redox signalling via uptake of NOX-derived H_2O_2 and in cellular mechanisms for minimizing oxidative stress.

A recent review of H_2O_2 permeability of AQPs suggested that rat AQP1 may be permeable to H_2O_2 whereas human AQP1 is not [134]. The authors of the cited study [127] suggest that the difference is due to differences in plasma membrane localization, although surface expression was not measured directly. This difference is particularly interesting given the speculation that we made regarding differences in CO_2 permeability between human and mouse AQP1 and the possible differences in AQP8 urea permeability between species and raises the important point that despite high levels of conservation between AQPs from different mammals, they are not exactly the same proteins (e.g. rAQP1 and hAQP1 differ by 18 residues), and direct comparison between AQPs from different mammals in different experimental systems may not always be appropriate.

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5. The physiological role of AQP6: An unusual AQP

AQP6 is expressed primarily in kidney epithelia, where it is expressed only in intracellular membranes. Immunostaining was observed in podocyte intracellular vesicles, sub-apical vesicles in straight proximal tubule cells, and in both sub-apical and sub-basolateral domains within type A (acid-secreting) intercalated cells in the collecting duct[135].

AQP6 has low intrinsic water permeability: The membrane water permeability of *Xenopus* oocytes expressing AQP6 was increased less than threefold [22, 136], and in some experiments, not at all [25, 26]. In contrast to other members of the AQP family, mercury increases rather than inhibits AQP6 water permeability, which is reversibly increased approximately tenfold upon $HgCl_2$ application. Low pH activated a reversible anion permeability of AQP6 [22] which was also shown to be ~tenfold more permeable to nitrate than chloride [137]. In rat collecting duct, intercalated cells, AQP6 co-localises with H^+-ATPase in intracellular vesicles [22], and in the intercalated cells of alkali-loaded rats, AQP6 mRNA and protein levels increased after a week [138]. These results, along with the pH-activation of AQP6 water and anion permeability suggest involvement of AQP6 in acid-base balance in the renal collecting duct. However the lack of *in vivo* experiments with AQP6 knockouts or naturally occurring mutations precludes any meaningful conclusions about the physiological roles of AQP6.

6. Ion permeability of AQPs: An unresolved controversy

AQPs support bulk movements of fluid by giving high water permeability to membranes that secrete osmolytes. These osmolytes are often ions (e.g. Na^+, K^+ and Cl^-) and this is reflected in the fact that several AQPs form macromolecular complexes with ion channels and transporters [41]. Perhaps a more efficient way for nature to achieve this dual permeability would be to have both ions and water pass through the same channel. Indeed it has been observed that several ion channels can transport water [38], but it has also been suggested that some AQPs may function as ion channels.

The pH-sensitive ion permeability of AQP6 is well established, and it is likely that the monomeric pore is the ion pathway given that a point mutation to a pore-lining residue of AQP6 abolished ion permeability [137] and AQP6-mimicking mutations of a pore-lining amino acid residue of AQP5 conferred anion permeability [139]. There is also some evidence that the plant AQP nodulin-26 from soybean can act as a voltage-gated, anion-biased ion channel [140, 141].

More controversial is the idea that the fifth pore formed at the fourfold axis of the tetramer of AQP1 may function as a cation channel. In *Xenopus* oocytes, heterologous expression of human AQP1 led to a PKA-activated cation permeability probably mediated by phosphorylation of AQP1 [142] and a cGMP-activated cation permeability via direct binding of cGMP to the C-terminus of AQP1 [143]. PKC activity was shown to increase the cation permeability of AQP1 in *Xenopus* oocytes via direct phosphorylation of AQP1 at residues T157 and T239 [144]. We recently showed that phosphorylation at these sites by PKC is required for trigger-induced translocation of AQP1 to the plasma membrane [145], so this may represent more AQP1 molecules in the membrane rather than an increase in the cation permeability or open probability of a single channel. Phosphorylation of a tyrosine residue (Y253)

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in the C-terminal tail of AQP1 was recently shown to be required for activation by cGMP [146]. The cGMP-activated cation current was verified for purified AQP1 in planar artificial membranes and the AQP1 water permeability inhibitor pCMBS did not inhibit ion permeation, suggesting that the water and ion pathways through AQP1 are not the same[147]. Furthermore, point mutations to residues lining the tetrameric pore altered conductance properties [146]. The open probability was found to be very small ($<10^{-6}$) in the planar bilayer system [147], which raises doubts over the physiological relevance of this cation conductance. This study suggested that this very low probability could reflect a misfolding or protein degradation artifact rather than a "real" function of AQP1. Furthermore, human APQ1 expressed in HEK293 cells did not induce an above-background cation conductance when cells were loaded with either cGMP or analogues thereof [148]. Additionally, after the initial report of AQP1 cation permeability by Yool et al, several laboratories reported being unable to replicate this result in the same experimental system (and in some cases, with exactly the same AQP1 construct) [149]. High variability in oocyte response to forskolin was also reported. It was suggested that the discrepancy could be due the protocol for choosing 'healthy' oocytes on which to perform experiments. A cut-off for oocyte membrane potential is routinely used for this. Yool et al choose oocytes with a potential < -20 mV, whereas others used a -35 mV cut-off. If AQP1 does indeed act as a cation channel it is plausible that it could cause a slight depolarization, leading to oocytes in which AQP1 is acting as an ion channel to be falsely excluded as unhealthy.

Rat choroid plexus cells, which strongly express AQP1, were found to have a cGMP-activated cation conductance that was abolished by treatment with AQP1 siRNA. Activation of the current by atrial natriuretic peptide (ANP), which signals through guanylate cyclase, inhibited basal-to-apical fluid transport. The contribution of AQP1 to this inhibition was not confirmed directly, although Cd^{2+}, which was shown to inhibit cGMP-induced cation conduction of AQP1, reversed the inhibition of fluid transport [150]. ANP and a cGMP analogue were also shown to upregulate apical-to-basal fluid transport in cultured retinal pigment epithelial cells, but this was reversed by an inhibitor of AQP1 water permeability [151].

It is clear that, under the right set of circumstances, AQP1 can act as a cation channel, with the ion pathway probably residing in the central pore formed by tetrameric assembly. Whether or not this ion conductance has physiological relevance remains an open question. However, coupled water and ion movement are vital for several pathophysiological processes including tumour angiogenesis and cell migration [152] and epilepsy [153], so it remains an intriguing possibility that AQPs are performing a dual function.

7. Cell Volume Regulation by AQPs

Many cells have the ability to modulate their physical size. This is achieved by the import or export of osmolytes in order to move water into or out of the cell by osmosis. Regulatory cell volume decrease (RVD) is mediated by potassium chloride and taurine efflux and regulatory cell volume increase (RVI) by sodium influx [154]. AQPs play a role in mediating the osmotic water movement in cell volume regulation [155], but there is some evidence that their role may go beyond acting as a passive water pore.

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The stretch-activated transient receptor potential vanilloid type 4 (TRPV4) channel is a Ca^{2+}-biased non-selective cation channel (NSCC) that is activated by cell swelling[156] and has been implicated in cellular responses to osmotic stimuli[157]. In some cell types TRPV4 has been shown to provide a Ca^{2+} signal that is correlated with activation of the K^+ and Cl^- channels responsible for the decrease in cellular osmolality associated with RVD [157, 158]. In human and murine salivary gland cells, TRPV4 has a functional interaction with AQP5; in AQP5 knockout cells, the hypotonicity-induced calcium influx through TRPV4 was attenuated and subsequent RVD was abolished. Hypotonicity also increased cell surface expression of both TRPV4 and AQP5 and increased their co-localisation [159]. This suggests a role for AQP5 in the regulation of TRPV4 surface expression or hypotonicity-induced activity.

In another example, the RVD of sperm of AQP3 −/− mice was inhibited compared to wild-type mice and the mice displayed reduced fertility[160] [161]. Upon entry into the female reproductive tract, sperm normally encounter a decrease in extracellular osmolality, which is thought to be the signal that activates sperm motility[160]. However, this hypotonic stress also causes cell swelling which, if left uncorrected by RVD, leads to impaired fertilisation, likely due to excessive bending of the sperm tail inside the uterus [161]. If AQP3 was simply acting passively as a water channel, RVD would not be abolished in AQP3 −/− sperm but rather the timescale on which the cell reaches osmotic equilibrium would be increased. A possible explanation for reduced fertility and altered RVD in AQP3 −/− mice is therefore that AQP3, either alone or as part of a macromolecular complex which is disrupted by AQP3 knockout, is involved in the signalling pathway that activates RVD in sperm.

In a further example, when exposed to a hypotonic extracellular solution, cultured renal cortical collecting duct (CCD) cells, which do not endogenously express AQP2, swelled in proportion to the change in extracellular osmolality but did not exhibit RVD. However, when transfected with AQP2, these cells showed an RVD of approximately 40%. Shrinkage was mediated by Ca^{2+} influx through TRPV4, which activated Ca^{2+}-dependent K^+ and Cl^- channels and Ca^{2+}-dependent Ca^{2+} release from intracellular stores. In renal CCD cells expressing AQP2, hypotonic stress caused translocation of TRPV4 to the plasma membrane. This response did not occur in AQP2-negative cells. When TRPV4 was pre-translocated to the cell surface prior to hypotonic exposure, RVD was recovered in AQP2-null cells, showing that it is not simply the high water permeability of AQP2 that allows RVD. However, there did not appear to be any co-localisation between endogenous TRPV4 and overexpressed AQP2 in this system, either before or after hypotonic shock, indicating a functional rather than physical interaction [162]. These observations suggest that AQP2 forms part of a sensory and signalling pathway that results in TRPV4 translocation, possibly via sensing of extracellular osmolality.

In a nasopharyngeal cancer cell line, swelling-induced chloride currents could be inhibited by siRNA-mediated knockdown of AQP3, extracellular application of $CuCl_2$ (a non-specific AQP3 inhibitor) or injection of AQP3 antibodies via recording pipettes. AQP3 also co-immunoprecipitated with the ClC3 chloride channel in this system [163]. The effect of the current-inhibiting treatments on AQP3-ClC3 interaction or ClC3 subcellular localization was not investigated, but this data

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suggests a role for AQP3 in RVD chloride efflux in these cells, possibly mediated via activation or localization of the ClC3 chloride channel.

It has been suggested that AQPs could act as direct sensors of osmotic gradients by coupling conformational changes of the protein to a pressure gradient within the pore [164]. This argument relies on the idea of the hydrostatic pressure within the pore and it is not clear that the application of classical fluid mechanics is applicable to a system typically consisting of <10 water molecules. Nonetheless it is an intriguing idea, and taken together, these examples clearly support the idea of a signalling or sensory role for AQPs in cell volume regulation beyond a passive water conduction mechanism.

8. Regulation of membrane protein localization by AQPs

There is some evidence that the expression of one AQP can regulate the expression, localization or membrane trafficking of other AQPs and of other membrane proteins.

AQP3 -/- mice have polyuria and a urinary concentrating defect. In the cortical collecting ducts of knockout mice, apical AQP2 expression was reduced compared to controls and basolateral AQP4 localization was completely absent. In contrast, medullary collecting duct levels of both AQP2 and AQP4 was not different between wild-type and AQP3 -/- mice [105]. This may reflect a requirement of AQP3 for expression or trafficking of AQPs 2 and 4 in certain cell types, although these observations could also be due to regulation of AQPs 2 and 4 in response to the polyuria phenotype.

AQP11 -/- mice develop polycystic kidney disease (PKD). In AQP11 -/- mice, polycystin 1 (PC1), a protein to which loss of function mutations are associated with autosomal dominant PKD, was upregulated twofold. However, the plasma membrane localization of PC1 was almost completely abolished and PC1 was differentially glycosylated in the AQP11 -/- mice compared to AQP11 +/+ [165]. Glycosylation of some membrane proteins is a crucial step in protein folding and passage through the ER quality control system [166]. Other membrane proteins (AQP1 and PC2) were correctly glycosylated, suggesting that this was a PC1 specific effect. This data suggests that AQP11 can regulate the surface expression of PC1, possibly by mediating an interaction with a glycosyltransferase or part of the ER quality control system.

The interactions between the stretch-activated cation channel TRPV4 and both AQP2 and AQP5 has been discussed in section 6. Briefly, expression of these AQPs was required for the hypotonicity-induced translocation of TRPV4 to the plasma membrane associated with RVD [159, 162]. It may be that in these systems, AQPs and TRPV4 are present in the same vesicles, but the trafficking machinery interacts only with the AQP. We have recently described hypotonicity-induced translocation of AQP1 in HEK293 cells [145] and AQP4 in primary rat astrocytes [167], so this may reflect a general regulatory mechanism by which AQPs can mediate the subcellular localization of other membrane proteins.

The histone methyltransferase Dot1a was shown to inhibit the expression of AQP5 in murine renal cells. Knocking out Dot1a led to AQP5 expression in these cells. The total level of AQP2 expression was unchanged in the presence of AQP5, but AQP2 membrane trafficking was impaired. In addition to this, co-transfection of AQP5 and

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AQP2 into IMCD3 cells reduced AQP2 surface expression compared to transfection of AQP2 alone, suggesting that AQP5 can regulate AQP2 localization. Furthermore, AQP2 and AQP5 co-immunoprecipitated with one another, suggesting a direct physical interaction [168]. The stoichiometry of the AQP2/AQP5 complexes was not investigated, so it is not clear whether the interaction was between AQP2 and AQP5 homotetramers, or through the formation of AQP2/AQP5 heterotetramers (which is an established regulatory mechanism employed by some plant AQPs [169]).

9. AQPs and cell-cell adhesion

Adhesion of cells to one another is a vital process that paved the way for evolution of multicellular organisms, and allows for the formation of the varied and complex structures that make up the anatomy of modern animals and plants.
There is evidence that some AQPs can perform a cell-cell adhesion function by forming a direct interaction with a membrane component on an adjacent cell (possibly another AQP molecule). This is well established for AQP0 and there is controversial evidence that AQP4 may also be able to perform this function.

9.1 AQP0

AQP0 (also called MIP, for lens Membrane Intrinsic Protein) is expressed primarily in lens fiber cells, which are cells of the eye specialized to form a tightly-packed transparent layer that scatters a minimal amount of incident light to support the function of the eye [8]. It is the most highly expressed membrane protein in these cells (45% by molarity of all lens fiber membrane protein in mice [170]). AQP0 tetramers assemble into large arrays in junctional microdomains [171], which dynamically associate and dissociate at the array edges, with only an estimated 1% existing as free tetramers at a given time, measured by high-speed atomic force microscopy of isolated sheep lens fiber membranes [172].

Knockout studies and a variety of naturally-occurring AQP0 mutations in humans and mice [8] consistently show that loss of AQP0 function in the lens fiber cells causes cataracts, likely via loss of the tight packing of the cells required to minimize light scattering. It has also been suggested, based on analysis of lenses of AQP0 -/- and -/+ mice, that AQP0 is vital for creating the lens refractive index gradient required to minimize spherical aberration, via an unknown mechanism [173].

A qualitative analysis of lens fiber cell-cell adhesion in AQP0 -/- mice showed that transgenic expression of AQP1 in the AQP0 -/- model could not rescue fiber adhesion [4], nor correct lens transparency defects [174], supporting the idea that the role of AQP0 in lens physiology is not related to membrane water permeability. Furthermore, expression of AQP0 in several i*n vitro* model systems consistently show that it is able to mediate cell-cell adhesion [175].

Early biophysical work on AQP0 proteoliposomes showed that AQP0 is able to mediate interactions with membranes containing negatively charged lipids [176]. It was suggested that this is due to the presence of several positively charged (and no negatively charged) residues in the extracellular loops A, C and E of AQP0. In human AQP0 this positively charged surface is made up of two arginine residues (R33 and R113) and three histidine residues (H40, H122 and H201).

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A naturally occurring AQP0 mutation, R33C, showed a reduction in cell-cell adhesion of 18, 27 or 34% (depending on methodology) for the mutant when compared to wild-type AQP0, despite no effect on membrane localization [177]. This suggests that removal of the conserved positive charge in loop A has a deleterious effect on AQP0-mediated cell adhesion, supporting the idea of a positively charged face of AQP0 that forms electrostatic interactions with negatively charged molecules on an adjacent membrane. It is also possible that addition of the cysteine sulfhydryl group facilitates a novel post-translational modification that inhibits adhesive interactions.

Crystallographic analyses of AQP0 suggest a different model of AQP0 mediated cell-cell adhesion in which AQP0 molecules on adjacent cells interact with one another directly. An electron crystal structure of double-layered 2D AQP0 crystals suggested that two AQP0 molecules on adjacent membranes might be able form an interaction via their extracellular loops, primarily mediated by proline residues [178]. Follow-up work showed that the way in which the AQP0 extracellular domains (ECDs) interact in these crystals is strongly dependent on the headgroup of co-crystallized lipids [179]. It has been suggested, based on comparison of the electron structure with a 3D X-ray structure of full-length AQP0 [180], that a conformational change in loop A induced by proteolytic truncation of the C-terminus (which is observed *in vivo* [8]) could facilitate direct binding of AQP0 molecules to one another [181]. However biochemical analysis showed that truncation of either the N- or C-terminus has no effect on the adhesion properties of AQP0 [182].

Experiments with a fusion protein consisting of the sequences of the three extracellular loops of AQP0 fused directly adjacent to one another showed that this protein was able to competitively inhibit AQP0 mediated cell-cell adhesion [183]. Given the relative location of the three loops in the AQP0 fold and the lack of transmembrane anchoring in the fusion protein, it is very unlikely that this protein is structurally similar to the ECD of intact AQP0. The fact that, despite this, it functions as an inhibitor of AQP0 mediated cell-cell adhesion does not support the idea of the adhesion being mediated by direct AQP0-AQP0 binding, which would depend on the structure of the ECD. It does, however, support the idea of mediation via an (structure independent) electrostatic interaction between the positively charged AQP0 ECD and a negatively charged membrane component on an adjacent cell.

9.2 AQP4

AQP4 is a water-selective AQP primarily expressed in glial cells (astrocytes and retinal Müller cells) of the central nervous system and the basolateral membranes of kidney collecting duct cells [155]. AQP4 exists in two major isoforms (although there is emerging evidence of further isoforms [184]). These are the long (323 amino acids) M1 isoform and the shorter (301 amino acids) M23 isoform, named for the position of the N-terminal methionine. Similarly to AQP0, the M23 isoform of AQP4 is able to form large supramolecular arrays (termed OAPs for Orthogonal Array of Particles).

Electron crystallography of double-layered 2D AQP4-M23 crystals suggested that AQP4 molecules on adjacent cells could interact via their ECDs in a similar way to that suggested for AQP0, again mediated by interaction between proline residues [185]. In the same study, AQP4-M23 was shown to facilitate cell-cell interactions between adhesion-deficient mouse fibroblasts. This result was corroborated in a

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similar experimental system, and it was shown that the adhesion generated by AQP4-M23 was considerably weaker than that by AQP0. Furthermore, AQP4-M1 was shown not to facilitate adhesion [175]. Another study in the same experimental system failed to reproduce these results and furthermore failed to find AQP4-mediated adhesion in primary mouse glia or AQP4-transfected CHO cell membrane vesicles [186].

Transfection of AQP4-M23 into a glioblastoma cell line (D54, which lacks endogenous AQP4 expression) increased adhesion of the cells to collagen, fibronectin, laminin, and vitronectin substrates [187]. Clearly this is not due to any potential cell-cell adhesion properties of AQP4 and the lack of substrate specificity makes this a difficult result to interpret. However, AQP4-M23 was shown to preferentially localize to membrane-substrate adhesion sites [188], so it is possible that AQP4-M23 could facilitate aggregation or localization of adhesion molecules, thereby having an indirect effect on cellular adhesion, both to other cells and to basement membrane components.

10. Conclusion

Mammalian AQPs are primarily known as facilitators of physiological processes through bi-directional, passive water transport. We have reviewed evidence demonstrating that this is far from all they do. AQPs facilitate physiological processes by mediating the diffusion of small, neutral solutes. This is best understood for AQP-mediated glycerol transport that facilitates glycerol metabolism, triglyceride cycling and skin hydration. AQPs also have a role in cell volume regulation and it is becoming increasingly clear that this role can extend beyond simply acting as a passive water pore and may involve AQP-mediated signal transduction, although the mechanisms that may be involved in this are not well understood. Many cell types express several different members of the AQP family and there is emerging evidence that the different members can interact with one another either physically or functionally. The involvement of AQPs in human physiology and cellular homeostasis goes far beyond their acting as simple water pores with implications for a range of physiological processes and pathophysiological conditions.

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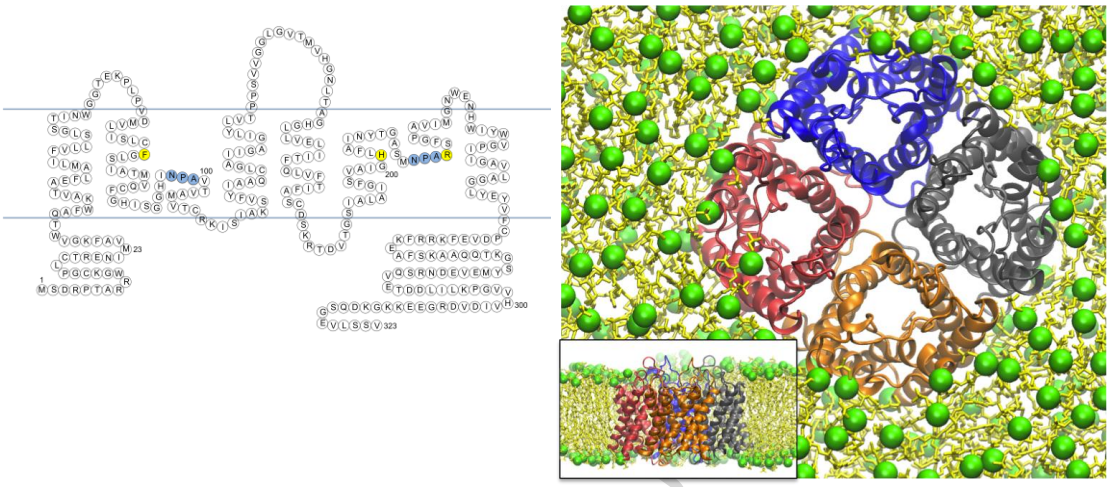

Figure 1 – structure of AQPs exemplified by human AQP4. (A) AQPs have six bilayer-spanning helices and two helix-forming loops. These loops contain the conserved NPA motifs (coloured blue). The residues forming the arginine/aromatic region thought to be important for solute selectivity are coloured yellow. (B) Simulation snapshot of AQP4 embedded in a POPC bilayer. Phosphorus atoms in the lipid headgroups are coloured green and the fatty acid tails are yellow. Visualized using VMD[189].

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Beyond water homeostasis: Diverse functional roles of mammalian aquaporins

Highlights

- AQPs are crucial for mammalian glycerol homeostasis
- AQPs may form part of a system for urea transport with built-in redundancy
- There is controversial evidence for gas and ion transport by AQPs
- AQP6 is an unusual AQP whose physiological role is unknown
- AQP function in cell volume regulation involves more than just water permeability

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